

## Gene cloning and heterologous expression of pyranose 2-oxidase from the brown-rot fungus, *Gloeophyllum trabeum*

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**Abstract** A pyranose 2-oxidase gene from the brown-rot basidiomycete *Gloeophyllum trabeum* was isolated using homology-based degenerate PCR. The gene structure was determined and compared to that of several pyranose 2-oxidases cloned from white-rot fungi. The *G. trabeum* pyranose 2-oxidase gene consists of 16 coding exons with canonical promoter CAAT and TATA elements in the 5'UTR. The corresponding *G. trabeum* cDNA was cloned and contains an ORF of 1,962 base pairs encoding a 653 amino acid polypeptide with a predicted molecular weight of 72 kDa. A Hisx6 tagged recombinant *G. trabeum* pyranose 2-oxidase was generated and expressed heterologously in *Escherichia coli* yielding 15 U enzyme activity per ml of induced culture. Structural alignment and phylogenetic analysis were performed and are discussed.

**Keywords** Gene cloning · *Gloeophyllum trabeum* · Glucose oxidase · Pyranose 2-oxidase

### Introduction

The enzyme, pyranose 2-oxidase (P2Ox) (EC 1.1.3.10), catalyzes the oxidation of several aldopyranoses and disaccharides at the C-2 position yielding the

corresponding 2-keto sugars (Giffhorn 2000). In addition to O<sub>2</sub> serving as an electron acceptor, P2Ox can also use a number of quinones and metal ions as electron acceptors. The specific activity of P2Ox varies considerably depending on the mono or disaccharide substrate used, with D-glucose as the preferred substrate. Given the range of both potential substrates and electron acceptors, P2Oxs show potential for several biotechnological applications including carbohydrate modification, fine chemical production, antibiotics, and as a bio-element in sensors and biofuel cells (Tamaki et al. 2007). These enzymes are FAD-dependent and harbor a number of highly conserved features including the FAD-binding site consisting of four separate subregions, a flavin attachment loop, and a C-terminal substrate binding domain. Several P2Oxs have been isolated, cloned and characterized from a number of white-rot fungi (de Koker et al. 2004; Leitner et al. 1998; Vecerek et al. 2004; Danneel et al. 1993). While activity in brown-rot fungi, no corresponding gene has yet been isolated and characterized (Volc et al. 1985).

The purpose of this study was to identify a pyranose 2-oxidase gene in the representative brown-rot fungus, *Gloeophyllum trabeum*, compare this to the several known P2Oxs from white-rot fungi, and engineer a cDNA for heterologous expression of this enzyme. We prepared degenerate nucleotide primers from several highly conserved regions of white-rot fungi P2Ox and amplified a small region of the *G. trabeum* P2Ox gene. Starting from this segment genome

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walking was performed, the full gene was isolated, and appropriate primers were used to isolate a full-length cDNA from purified mRNA. Recombinant P2Ox was expressed in *E. coli* and the enzymatic activity was demonstrated.

## Materials and methods

### Microorganisms

*Gloeophyllum trabeum* MAD-617 (ATCC 11539) was used for this study. *E. coli* DH5 $\alpha$  and BL21(DE3) (Novagen) were used as hosts for DNA manipulation and recombinant protein expression, respectively.

### Genomic DNA purification

A mycelial fragment was inoculated into 50 ml malt extract broth in a 1 l Erlenmeyer flask and incubated at 28°C and 100 rpm for 7 days. Mycelia were harvested by filtration through Miracloth (Calbiochem) snap-frozen in liquid N<sub>2</sub> and ground to a fine powder using a mortar and pestle. The ground mycelia were suspended in extraction buffer [200 mM Tris/HCl pH 8.5, 250 mM NaCl, 25 mM EDTA, 0.5% (w/v) SDS] and extracted twice by mixing thoroughly with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, by vol.), incubated on ice for 15 min, and centrifuged at 4,000 $\times g$  for 45 min. The recovered aqueous phase was supplemented with 50  $\mu$ l 10 mg RNase A/ml and incubated at 37°C for 10 min followed by 56° for 15 min. Proteinase K was then added at 0.5 mg/ml and the mixture held at 56°C for 3 h. Nucleic acid was then precipitated by addition of an equal volume of 2-propanol, washed in 70% (v/v) ethanol, and resuspended in an appropriate volume of TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0).

### Gene cloning of *G. trabeum* P2Ox

The P2Ox gene from *G. trabeum* was isolated by redundant PCR using 200 ng genomic template, 200 pmol each of redundant primers 5'-GGZGGZA TGKCZACNCAYTGGACN-3' (forward, 64-fold complexity) and 5'-CIARICCIGGITCCATRAAYT GNGG-3' (reverse, 32-fold complexity) corresponding to highly conserved regions in the substrate

binding domain of known P2Ox as indicated (Fig. 1). Thermal cycling was at 94°C for 2 min followed by 40 cycles of 94°C for 45 s, 53° for 45 s, 72° for 2 min with a final 7 min extension at 72°C. Products were gel purified, recovered using solid phase extraction (Qiagen), A-tailed and cloned into pGemTeasy (Promega) for DNA sequencing according to the manufacturer's instructions. Based on this P2Ox gene fragment, appropriate specific primers were generated iteratively and used with the Universal Genome Walker kit (Clontech) to recover the full gene. Subsequence genomic DNA purification for overlapping sequence confirmation was performed using the ZR Genomic DNA Kit (Zymo Research). All sequencing reactions were performed using the BigDye Terminator kit (Applied Biosystems) according to the manufacturer's instructions and analyzed at the University of Wisconsin Biotechnology Center.

### cDNA purification and cloning

Mycelia were prepared as described above and RNA was recovered using a Dynabead extraction kit (Invitrogen) and further purified using the Oligotex mRNA kit (Qiagen). First strand synthesis was performed using an Accuscript Kit (Stratagene) according to the manufacturer's instructions. *Gloeophyllum trabeum* P2Ox was amplified from this cDNA using *Pfu* proofreading DNA polymerase (Stratagene) with primers 5'-ATGTCCCTCAGGCC TGATGAC-3' (forward) and 5'-CCGTAATCTCA GGTCCCTCTGACA-3' (reverse). The product was gel purified and cloned into pCR-Blunt (Invitrogen) and sequenced as described above.

### Heterologous expression of *G. trabeum* P2Ox

The *G. trabeum* P2Ox cDNA was amplified using primers 5'-CGCGGATCCATGTCCCTCAGCC-3' (forward) and 5'-CAGAAGTTGCGGCTCC CG AATCCAAGC-3' (reverse) which introduce *Bam*H1 and *Hind*III restriction sites, respectively as indicated by underlining. This fragment was introduced into the *Bam*H1-*Hind*III interval in pET21a(+) (Novagen) resulting in an in-frame fusion with the *N*-terminal T7-tag and the *C*-terminal His<sub>6</sub>tag. The resulting plasmid was transformed into *E. coli* BL21(DE3) for expression. A fresh colony was inoculated into 25 ml Terrific Broth (TB) supplemented with 100  $\mu$ g

GtP2Ox	-----MSLSPDPP-----FFGFDNGGWLSKPARALADHATRAGVFGYAPAALSLPPLRLLPDPGVPEGIPKYDIVIA	72
PgP2Ox	-----MSASSSDP----FHSFAKTSFTSKAAKRAT-----AHSLPPLP-GPGDLPPGMNV-EYDVAIV	56
ThP2Ox	-----MSASSSDP----FHSFAKTSFTSKAAKRAT-----AHSLPPLP-GPGDLPPGMNV-EYDVAIV	56
TpP2Ox	-----MSTSSSDP---FYNFAKTSFKSAAAQKAS-----ATSLPPLP-GPDQKVPGMDI-KYDVVIV	56
TvP2Ox	-----MSTSSSDP---FFNFTKSSFRSAAAQKAS-----ATSLPPLP-GPDKKVPGMDI-KYDVVIV	56
ToP2Ox	-----MSTSSSDP---FFNFAKSSFRSAAAQKAS-----ASSLPPLP-GPDKKVPGMDI-KYDVVIV	56
PsP2Ox	-----MSTSSSDP---FFNFAKSSFRSAAAQKAS-----ASSLPPLP-GPDKKVPGMDI-KYDVVIV	56
PcP2Ox	-----MLDTPTPRAD-----EP-YDVFIA	23
EnP2Ox	MOYSRMTATRENPKYKNLRVEECVLKSGSTTNP-----R-----IC-----G1KLNNNSTV	51
TmP2Ox	-----MPIRLSKE-----KINDLQLRSQCDLTSS-----Q-----DE-----IVHYTDVFIA	41
GtP2Ox	IGATYARLEVEAGF-----NVAMPDVGEIDSGPKR-----GSHKKNAIEYQKNIIDKFVH	121
PgP2Ox	IGSTYARLEVEAGF-----NVAMPE1GEIDSGLK1-----GSHKKNTVEYQKNIIDKFVN	105
ThP2Ox	IGCTYARLEVEAGF-----NVAMPE1GEIDSGLK1-----GSHKKNTVEYQKNIIDKFVN	105
TpP2Ox	IGCTYARLEVEAGY-----KVAMPD1GEIDSGLK1-----GAHKKNTEVYQKNIIDKFVN	105
TvP2Ox	IGCTYARLEVEAGY-----KVAMPD1GEIDSGLK1-----GAHKKNTEVYQKNIIDKFVN	105
ToP2Ox	IGCTYARLEUVGAGY-----KVAMPD1GEIDSGLK1-----GAHKKNTEVYQKNIIDKFVN	105
PsP2Ox	IGCTYARLEUVGAGY-----KVAMPD1GEIDSGLK1-----GAHKKNTEVYQKNIIDKFVN	105
PcP2Ox	IGATAFKLCVDANL-----RVCMEVGAADSFSTKPMKGDPNAPRSVQFGPGQVPIPGYHKRNIEYQKDIDRFVN	94
EnP2Ox	VGATYAREILDPGSGASPGRKAPKVIMVE1GAQESKVP-----GEHKRKNAVVYQKHIDSFVN	108
TmP2Ox	IACTYARHIDNTS-----TTKVYMAE1GSQDNPVI-----GAHRRNSIKPKQKDTDKFVN	91
GtP2Ox	VIQGQLMPVSPVINKLVADTLSP-----SSWQASRHFVRNASNPQNPFRLNQQAVTRV	193
PgP2Ox	VIQGQLMPVSPVNTMVDTLSP-----ASWQASTFFVRNGANPQEQLPDRNLSSQAVTRV	177
ThP2Ox	VIQGQLMPVSPVNTMVDTLSP-----ASWQASTFFVRNGANPQEQLPDRNLSSQAVTRV	177
TpP2Ox	VIQGQLMSVSPVNLKLVADTLSP-----TSWQASTFFVRNGSNPQEQLPDRNLSSQAVTRV	177
TvP2Ox	VIQGQLMSVSPVNTLVIDLTLSP-----TSWQASSTFFVRNGSNPQEQLPDRNLSSQAVTRV	177
ToP2Ox	VIQGQLMSVSPVNTLVIDLTLSP-----TSWQASTFFVRNGSNPQEQLPDRNLSSQAVTRV	177
PsP2Ox	VIQGQLMSVSPVNTLVIDLTLSP-----TSWQASTFFVRNGSNPQEQLPDRNLSSQAVTRV	177
PcP2Ox	VIKGALSTCS1PTSNNHIATLDPVS-----VSNSLDPKF1ISLGKPNPAQNPVFNVLGAEAVTRGV	171
EnP2Ox	EKQ-----NFN-----GQNKEQNIYHNLDANGVSRNV	151
TmP2Ox	IINGALQPI1S1PSD1TYQPTLAVA AAWAPP1DPAEGQLVIMGHNPQNEAGLNLPGSATRTRV	168
GtP2Ox	-----MERPKLVRDDNAADDEEWNRLYREAEQF1ATGHSQFERS1RHTLVLLETLRDSYNGT--RAFEQ1PLAATRTDPD	265
PgP2Ox	-----LQRPLLVKNDPVADDAEWDRLYKKAESYFK1GTTQFAES1RHLNVLKLLQEEYKGV--RDFQO1PLAATRQSP	248
ThP2Ox	-----LQRPLLVKNDSKADDAEWDRLYKKAESYFK1GTTQFAES1RHLNVLKLLQEEYKGV--RDFQO1PLAATRQSP	248
TpP2Ox	-----EQRPLLVKDPPDADD1IWDLYT1KAESYFK1GTTQFAES1RHLNVLKLLQEEYKGQ--RTFQO1PLAATRRNP	248
TvP2Ox	-----EQRPLLVKDQQDADD1EWDRLY1KAESYFK1GTTQFAES1RHLNVLKLLQEEYKGQ--RDFQO1PLAATRSP	248
ToP2Ox	-----EQRPLLVKDADDAEWDRLY1KAESYFK1GTTQFAES1RHLNVLKLLQEEYKGQ--RDFQO1PLAATRSP	248
PsP2Ox	-----EQRPLLVKDADDAEWDRLY1KAESYFK1GTTQFAES1RHLNVLKLLQEEYKGQ--RDFQO1PLAATRSP	248
PcP2Ox	FNAPHRERPKLS-TDAEEDAR1WKDLYAQAKE1I1GTTTEFDHS1RHLNVLRKYND1FQKENVIREFSPLPLACHRLTD	250
EnP2Ox	-----LER----SK-IFDDATWDRLYKRAEEL1GTRTDVLDQSQ1RQLVLD1LRRKFKN----RDAKALPLAEEK-VEG	215
TmP2Ox	-----EER----VNNPVDKQEFDALLERAKTLINLVHSDQYDDSTRQ1VVEKTLQQLTDAS----RGVTTLPLGVERRTDN	235
GtP2Ox	SQFIQWSAHT1VFDLEDRPNTTHPGERF1-----PGVMCTQV1-----RNSENTA1K1STRVQDVTPN-----SAP-----VEI	331
PgP2Ox	-TFVEWSSAHT1VFDLENRPKNDAPKQRFN1-----PAVACTSVR-----RNDANEII1GLDVRDLHG-----GKS-----ITI	311
ThP2Ox	-TFVEWSSAHT1VFDLENRPKNDAPKQRFN1-----PAVACTSVR-----RNDANEII1GLDVRDLHG-----GKS-----ITI	311
TpP2Ox	-TFVEWSSANT1VFDLQNRPNTDAPNERF1-----PAVACERVM-----RNSA1NTAI1ESL1HIDLIS-----GDR-----FAI	311
TvP2Ox	-TFVEWSSANT1VFDLQNRPNTDAPNERF1-----PAVACERVM-----RNTSNSEIESL1HIDLIS-----GDR-----FEI	311
ToP2Ox	-TFVEWSSANT1VFDLQNRPNTDAPNERF1-----PAVACERVM-----RNLAINSEIESL1HIDLIS-----GDR-----FEI	311
PsP2Ox	-TFVEWSSANT1VFDLQNRPNTDAPNERF1-----PAVACERVM-----RNLAINSEIESL1HIDLIS-----GDR-----FEI	311
PcP2Ox	-DYVEWHAETDRILEELQNPRTDPVKR--GRFT1TNHRCRKT1LKFVHYRGEENEYDVALVEDLPHMNPQNPASV---KKI	323
EnP2Ox	KNLIKWSSSTSTVGNLNLLEDE-K-----FTLDQHCKLEF1N-----DET--NKVSFA11K1LAKPQ-----TSKEDEDRLR	279
TmP2Ox	PIYV1WTWGTADTVLGVDPVKSP-----RFV1VETRVTKF1IVS-----ETNP-TQVVA1LLRNLN-----TSN-----DELV	294
GtP2Ox	SADFV1F1LVT1-----AVHNPO1-----VNSGF-----KLGRPDASDANEVG1L1PFL1-----SF1ITEQ1LAFCQ1VLSKELVNN	397
PgP2Ox	KAKVY1ILT1-----AVHNQ1-----AASGF-----QLGRPDPAK1P1-SLLP1-----WHITEQ1L1VFCQ1VMS1ELINS	376

**Fig. 1** Alignment of *G. trabeum* MAD-617 P2Ox (GtP2Ox; accession ACJ54278) translated from cDNA with P2Ox from *Peniophora gigantea* (PgP2Ox; accession GI:34452037), *Trametes hirsuta* (ThP2Ox; accession GI:25091016; Christensen et al. 1999), *Trametes pubescens* (TpP2Ox; accession GI: 57867849), *Trametes versicolor* (TvP2Ox; accession GI:25091018; Nishimura et al. 1996), *Trametes ochracea* (ToP2Ox; accession GI:31044224) *Peniophora* sp. (PsP2Ox; accession GI:274364221), *Phanerochaete chrysosporium* (PcP2Ox; accession AY522922; de Koker et al. 2004) and

*Trametes multicolor* (TmP2Ox; accession GI: 215794594; Leitner et al. 2001). Grey shaded amino acids represent identity to GtP2Ox. Solid lines above sequence indicate FAD-binding regions. Dotted line above sequence indicates flavin attachment loop, dashed line above sequence indicated substrate binding domain, underlined GtP2Ox sequence indicates regions successfully used for degenerate PCR primers, *down arrow head*; indicates intron locations and black shaded amino acids with white typeface indicates GMC-OXRED consensus residues (Albrecht and Lengauer 2003)

ampicillin/ml and incubated at 37°C and 150 rpm for 6 h. This culture was then used to inoculate 225 ml TB/ampicillin at 37°C and 150 rpm for an additional 2 h. These cultures were then supplemented with 5 g lactose/l and incubated at 25°C and 150 rpm for 16 h. Bacteria were harvested by centrifugation at 10,000×g

for 10 min at 4°C. The pellet was resuspended in 25 ml lysis buffer (50 mM KPO<sub>4</sub>, 1 M NaCl, 5 mM imidazole, pH 6.5) and disrupted by two passages through a French press at 100 mPa. Lysates were clarified by centrifugation at 30,000×g for 30 min at 4°C and filtered through 0.45 µm PVDF membranes (Millipore).

GtP2Ox	KAKVYILTAG-AVHNQLEAASGF	-QLGRPDPAKPLP-SLLPYL	GTHITEQTLVFCQTVMSTELINS	376
TpP2Ox	QADVVVLTAG-AVHNQLEVNNSGF	-KLGRPDPAKP-P-ELLPF	GSYITEQSLVFCQTVMSTELIDS	375
TvP2Ox	KADVFVLTAG-AVHNQLEVNNSGF	-QLGRPDPAKP-P-ELLPF	GSYITEQSLVFCQTVMSTELIDS	375
ToP2Ox	KADVFVLTAG-AVHNQLEVNNSGF	-QLGRPDPAKP-P-ELLPF	GSYITEQSLVFCQTVMSTELIDS	375
PsP2Ox	KADVVVLTAG-AVHNQLEVNNSGF	-QLGRPNPTNP-P-ELLPF	GSYITEQSLVFCQTVMSTELIDS	375
PcP2Ox	YARSYVVACG-AVATAQVANSHI	PPDDVVIPFPGEKGSGGGGERDATIPTPLPM	GKYITEQPMTCQVVLDSLMEV	402
EnP2Ox	IKAKVVIWCGPILTPOLPKSGFRYD	-EEDAEDSEGNKSSLYI	PAQRNLTEQTMCFQCQIVLKDKNVVE	348
TmP2Ox	VAQSFVIACG-AVCTPQILVNNSNRP	-	HALCRYLSSEQSMTCQCIVLKRSTIVDS	345
GtP2Ox	VKADMFRVSQTGPQDPDYKVEWTGDPANKHPDWNEKEVKHHMHEHQ-	EDPLPITPLHDEPEQVITLFEQSHPWHTQIHRDA	-	475
PgP2Ox	VTDADMIVGKPGPDPYSVTYTSGSPPNNKHDPDWNEKEVKHHMHDHQ-	EDPLPITPLHDEPEQVITLFEQSHPWHTQIHRDA	-	454
ThP2Ox	VTADMTIVGKPGPDPYSVTYTPGPNPNNKHDPDWNEKEVKHHMHDHQ-	EDPLPITPLHDEPEQVITLFEQSHPWHTQIHRDA	-	454
TpP2Ox	VKSMDTIIGNPGELGYSVSMPGASTNKHDPDWNEKEVKHNHMQHO-	EDPLPITPLHDEPEQVITLFEQSHPWHTQIHRDA	-	453
TvP2Ox	VKSMDTIRGNPGDLGYSVTVTPGAETNKHPDWNEKEVKHNHMQHO-	EDPLPITPLHDEPEQVITLFEQSHPWHTQIHRDA	-	453
ToP2Ox	VKSMDTIRGTPGELTYSVTYTPGASTNKHDPDWNEKEVKHNHMQHO-	EDPLPITPLHDEPEQVITLFEQSHPWHTQIHRDA	-	453
PsP2Ox	VKSMDTIRGTPGELTYSVTYTPGASTNKHDPDWNEKEVKHNHMQHO-	EDPLPITPLHDEPEQVITLFEQSHPWHTQIHRDA	-	453
PcP2Ox	VR-----NPP-----WPG-----LDWWKEKVARHVEAPP-----NDPIPPIPRDPEPEQVITLFEQSHPWHTQIHRDA	-	-	459
EnP2Ox	LQKN-----NWGPECEEHRKYEDEDDPLRIFDLDLDPQVTLPTENTPWHTQIHRDA	-	-	401
TmP2Ox	IATD-----RFAAKVEAHKKHP-----DDVLPILFHEPEPQVMIPYTSDFPWHVQVHR--	-	-	395
GtP2Ox	FSYGAVABSIDTRLVVDWRFGRTRTEPEKEENKLWFSKO-	ITDQYGMQPQTDFRFPDGITSQADRMMTDMC	546	
PgP2Ox	FSYGAVQQSISDRSLIVDWRFGRTRTEPEKEENKLWFSDK-	ITDAYNLQPQTDFRFPBGR---EAEDMMTDMCV	522	
ThP2Ox	FSYGAVQQSISDRSLIVDWRFGRTRTEPEKEENKLWFSDK-	ITDAYNLQPQTDFRFPBGR---EAEDMMTDMCV	522	
TpP2Ox	FSYGAVQQSISDRSLIVDWRFGRTRTEPEKEENKLWFSDK-	ITDAYNMQPQTDFRFPAGRTSQAEADMMTDMCV	524	
TvP2Ox	FSYGAVQQSISDRSLIVDWRFGRTRTEPEKEENKLWFSDK-	ITDAYNMQPQTDFRFPAGRTSKAEADMMTDMCV	524	
ToP2Ox	FSYGAVQQSISDRSLIVDWRFGRTRTEPEKEENKLWFSDK-	ITDAYNMQPQTDFRFPAGRTSKAEADMMTDMCV	524	
PsP2Ox	FSYGAVQQSISDRSLIVDWRFGRTRTEPEKEENKLWFSDK-	ITDAYNMQPQTDFRFPAGRTSKAEADMMTDMCV	524	
PcP2Ox	FSYGAVBANEHMDTRIVDYRFYGTPEQANEELVFOOH-----YRDAYDMPQPTFKFTMSQDDR--ARARMMDMCN	-	-	529
EnP2Ox	FSYGAVPPAIDKRTIVDLRYFGRAETQWRNRNRTFSKK-----LTDAYGMQPQTDFKLSTKDR-LESRHMMDMKE	-	-	471
TmP2Ox	YAFGDVGPKADPDRVVDLRFKGSDIVEENRVTGPNPKLRDWEAGVTDYGMQPQTFHVKRTNADG-DRDQRMMNDMTN	-	-	474
GtP2Ox	MSSKIGGFLPGSNPQMEPGLVHLGGTH--RMG--FDEQED-KCCVDTDSKVFGFEN	FLGGCGNIE-TAYASNPTL	-	618
PgP2Ox	MSAKIGGFLPGSYQPQFMEPGLVHLGGTH--RMG--FDEKAD-KCCVDTDSRVFGFKN	FLGGCGNIE-TAYAANPTL	-	594
ThP2Ox	MSAKIGGFLPGSYQPQFMEPGLVHLGGTH--RMG--FDEKAD-KCCVDTDSRVFGFKN	FLGGCGNIE-TAYAANPTL	-	594
TpP2Ox	MSAKIGGFLPGSLQPQFMEPGLVHLGGTH--RMG--FDEQED-NCCVNTDSRVFGFKN	FLGGCGNIE-TAYGANPTL	-	596
TvP2Ox	MSAKIGGFLPGSLQPQFMEPGLVHLGGTH--RMG--FDEQED-NCCVNTDSRVFGFKN	FLGGCGNIE-TAYGANPTL	-	596
ToP2Ox	MSAKIGGFLPGSLQPQFMEPGLVHLGGTH--RMG--FDEKED-NCCVNTDSRVFGFKN	FLGGCGNIE-TAYGANPTL	-	596
PsP2Ox	MSAKIGGFLPGSLQPQFMEPGLVHLGGTH--RMG--FDEKED-NCCVNTDSRVFGFKN	FLGGCGNIE-TAYGANPTL	-	596
PcP2Ox	IALKIGGYLPGSEPQFMTPLGLALFLAGTT---RCG---LDTQ---KTVGNTHCKVHNPNFYVGNGNVIE-TGFAANPTL	-	-	599
EnP2Ox	VAGELGGYLPGSEPQFLAPGLALFVGTTAAALRKGCRSEDEMKRISVCDENSKVWGVENDLHGLGLNV	BGPSPNASNPTL	-	551
TmP2Ox	VANILGGYLPGSYQPQFMAPGLAQITGTT---RIG---TDDQ---TSVADPTSKVHNFDN	WVGNGCIE-DATACNPTR	-	544
GtP2Ox	TAMAMAIAKSCHEYIKKNFKPKSEIGSSDNRAWIRGAA	653		
PgP2Ox	TAMSLAIKSCHEYIKKNFEPSPSPNPVKHHN	622		
ThP2Ox	TAMSLAIKSCHEYIKKNFEPSPSPNPVKHHN	622		
TpP2Ox	TAMSLAIKSCHEYIKKNFEPSPFTPAQ	622		
TvP2Ox	TAMSLAIKSCHEYIKNNFTPSPFDTDQAE	623		
ToP2Ox	TAMSLAIKSCHEYIKQNFTPSPFPTSEAQ	623		
PsP2Ox	TAMSLAIKSCHEYIKQNFTPSPFPTSEAQ	623		
PcP2Ox	TSICYAIASNDIIAKFGRHRG	621		
EnP2Ox	TAMCFAIKGAEIIRRKLGKGGSHSGNRDDGVDTDDDA	591		
TmP2Ox	TSVAYALKGAEAVVSYLGVS	564		

**Fig. 1** continued

## Enzyme assay

Pyranose 2-oxidase activity was determined using the chromogen ABTS [2,2-azinobis(3-ethylbenzothiazoline-sulfonic acid)] ( $\epsilon_{420} = 43.2 \text{ mM}^{-1} \text{ cm}^{-1}$ ) (Danee et al. 1993). The standard 1 ml assay mixture contained 10  $\mu\text{mol}$  ABTS, 20 U horseradish peroxidase, 100  $\mu\text{mol}$  D-glucose, in 50 mM potassium phosphate buffer (pH 6.5). The reaction was started with the addition of 20  $\mu\text{l}$  diluted enzyme. Absorbance was measured at 420 nm and 25°C for 3 min. One unit of activity was defined as the amount of enzyme necessary for the oxidation of 2  $\mu\text{mol}$  ABTS per min (equivalent to the oxidation of 1  $\mu\text{mol}$  D-glucose) at 25°C.

## Bioinformatics

Multiple protein alignment was performed using the ClustalW algorithm with default settings supplied with Lasergene v8.0 software (DNAStar). Cladogram construction was performed using Treeview v1.6.6. (<http://taxonomy.zoology.gla.ac.uk/rod/rod.html>).

## Results and discussion

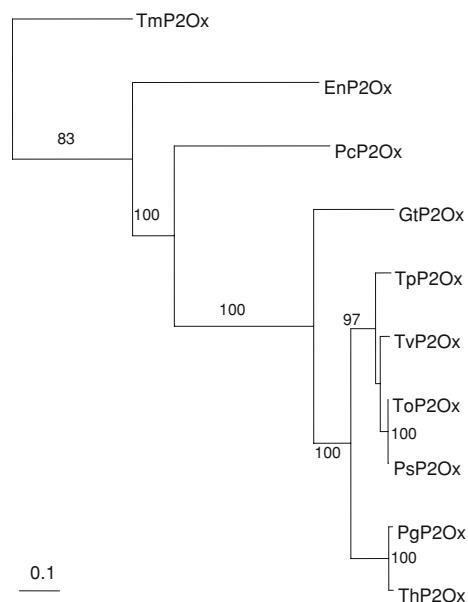
Redundant PCR using a number of PCR primer pairs generated against conserved regions of an alignment of known pyranose 2-oxidases (de Koker et al. 2004)

resulted in the successful amplification of a homologous DNA from *G. trabeum* using primers derived from the regions underlined in Fig. 1. Genome walking using an adapter library yielded the full-length gene. The *G. trabeum* P2Ox gene is predicted to be comprised of 16 coding exons with a predicted cDNA of 1,962 nucleotides coding for a polypeptide of 653 amino acids and a predicted molecular weight of 72 kDa. The P2Ox protein is not predicted to have a cleavable N-terminal secretory signal sequence.

A cDNA for *G. trabeum* P2Ox was isolated from polyA-selected RNA and found to correspond to the cDNA predicted from genomic material. A multiple sequence alignment shows that *G. trabeum* P2Ox conserves essential features of other known P2Ox including four regions of a predicted FAD-binding domain, predicted flavin attachment loops and a predicted substrate binding domain. Moreover, as described in a structural similarity analysis of *Trametes versicolor* (*Coriolus versicolor*) and *T. hirsuta*, *G. trabeum* P2Ox is conserved at 14 of the 15 identified strictly conserved residues for the GMC-OXRED family (Albrecht and Lengauer 2003) (Fig. 1). *Gloeoiphyllum trabeum* represents a phylogenetic intermediate between a cluster of closely interrelated P2Oxs including PgP2Ox (70% identity to GtP2Ox as determined by blastp, <http://www.ncbi.nlm.nih.gov>, see Fig. 1 for abbreviations) ThP2Ox (69% identity), TpP2Ox (70% identity), TvP2Ox (70% identity), ToP2Ox (70% identity), PsP2Ox (70% identity), and the comparatively less related (PcP2Ox 46% identity), and TmP2Ox (37% identity) (Fig. 2).

*Gloeoiphyllum trabeum* P2Ox was introduced into an *E. coli* expression system and analyzed in crude lysates and IMAC column purified fractions. A typical experiment resulted in 15 U pyranose-oxidizing activity per ml of induced heterologous expression culture. The recombinant P2Ox was recovered by IMAC chromatography and shown by SDS-PAGE to consist of a polypeptide of 72 kDa under reducing conditions as predicted from the translated cDNA (Fig. 3).

Given the considerable promise that pyranose 2-oxidase enzymes have in the production of fine chemicals, antibiotics, carbohydrate modification and biofuel sensors, the identification of novel P2Ox contributes to the pool of candidates for further characterization and improvement (Giffhorn 2000; Tamaki et al. 2007). As the first P2Ox isolated from a



**Fig. 2** Cladogram of P2Ox phylogeny. Distance analysis and tree construction was performed using the neighbor joining method of Megalign (DNAlign). Numbers within the tree indicate the percent support after 1,000 replications of bootstrap analysis. The bar represents 10% estimated sequence difference



**Fig. 3** Analysis of recombinant GtP2Ox. Commassie blue stain of approximately 0.5 µg of GtP2Ox in a reducing 10% SDS-PAGE gel showing the recombinant protein comigrating with a 72 kDa marker using EZ-Run Pre-Stained Rec protein ladder (Fisher) as indicated

brown-rot fungus, this enzyme appears to represent, from an identity standpoint, a midpoint between a tight cluster of described white-rot fungi P2Ox and a group of comparatively less related members. Further understanding of the unique characteristics of this P2Ox versus those described for a variety of white-rot fungi is a matter of ongoing investigation. As efforts to amend the characteristics of known P2Ox through rational design and directed evolution continue, the recovery of novel P2Ox provide another avenue to the identification of candidates with characteristics best suited to biotechnological utilization (Spadiut et al. 2008).

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